Review

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Molecular mechanisms of antimony resistance in *Leishmania*

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Leishmaniasis causes significant morbidity and mortality worldwide. The disease is endemic in developing countries of tropical regions, and in recent years economic globalization and increased travel have extended its reach to people in developed countries. In the absence of effective vaccines and vector-control measures, the main line of defence against the disease is chemotherapy. Organic pentavalent antimonials [Sb(V)] have been the first-line drugs for the treatment of leishmaniasis for the last six decades, and clinical resistance to these drugs has emerged as a primary obstacle to successful treatment and control. A multiplicity of resistance mechanisms have been described in resistant *Leishmania* mutants developed *in vitro* by stepwise increases of the concentration of either antimony [Sb(III)] or the related metal arsenic [As(III)], the most prevalent mechanism being upregulated Sb(III) detoxification and sequestration. With the availability of resistant field isolates, it has now become possible to elucidate mechanisms of clinical resistance. The present review describes the mechanisms of antimony resistance in *Leishmania* and highlights the links between previous hypotheses and current developments in field studies. Unravelling the molecular mechanisms of clinical resistance could allow the prevention and circumvention of resistance, as well as rational drug design for the treatment of drug-resistant *Leishmania*.

Overview

Leishmania, a protozoan parasite belonging to the family *Trypanosomatidae*, causes leishmaniasis, a group of diseases with clinical manifestations that range from self-healing cutaneous and mucocutaneous skin ulcers to a fatal visceral form [visceral leishmaniasis (VL) or kala-azar]. The disease is a significant cause of morbidity and mortality in several countries of the world. It is endemic in 88 countries, with approximately 400 000 new cases per year (Ashford *et al.*, 1992). Now, leishmaniasis is an emerging tropical disease in the USA (Rosypal *et al.*, 2003), with more than 500 parasitologically confirmed cases (Centers for Disease Control, 2004). Although VL (the lethal form of disease) has been reported in more than 60 countries (Sundar, 2001), nearly all of the 500 000 new cases annually of symptomatic VL occur in the rural areas of only five countries: India, Nepal, Bangladesh, Brazil and Sudan (Desjeux, 2001), with 50 % of all VL cases from the Indian subcontinent. The association of leishmaniasis with HIV has also been reported from 33 countries, with most cases in Southern Europe, where 25–70 % of adult patients with VL are coinfecting with HIV (Rosenthal *et al.*, 1995). It has been estimated that 1.5–9 % of patients with AIDS will develop leishmaniasis (Desjeux, 2000).

The *Leishmania* parasite is transmitted by an invertebrate sandfly vector, *Phlebotomus*. The parasite leads a digenetic

life cycle (Molyneux & Killick-Kendrick, 1987). Infected sandflies introduce the metacyclic forms of the promastigote stage into the bloodstream of the vertebrate host when they bite to take a meal. The promastigotes transform into an amastigote stage in the phagolysosome of reticuloendothelial cells, where they multiply in the hostile environment of the macrophage and kill the cell.

Since vaccines against leishmaniasis are still under development (Brandonisio & Spinelli, 2002), the control of the disease relies solely on chemotherapy. The organic salt of pentavalent antimony [Sb(V)] has been the first-line drug for all forms of leishmaniasis for more than 60 years (Herwaldt, 1999). However, in recent years, a large-scale increase in clinical resistance to pentavalent antimonials has been reported (Faraut-Gambarelli *et al.*, 1997; Lira *et al.*, 1999). In India, as many as 65 % of previously untreated patients fail to respond promptly or relapse after therapy with antimony drugs, due to the development of drug resistance (Sundar *et al.*, 2000).

Second-line drugs include pentamidine and amphotericin B, but severe side effects and high cost limit their use (Mishra *et al.*, 1992). Miltefosine (hexadecylphosphocholine), originally developed as a neoplastic agent, has now been approved as the first oral drug for leishmaniasis. It can be used for both antimony-responding and non-responding patients (Sundar *et al.*, 1999). Although it shows good

efficacy, it is very expensive and has a long life in the body. Preliminary data from phase IV clinical trials in India involving domiciliary treatment with miltefosine along with weekly supervision suggest a doubling of the relapse rate (Sundar & Murray, 2005). This provides a warning that resistance could develop quickly in the future, and therefore plans are required to prevent it. *In vitro* studies have established that a single point mutation may lead to miltefosine resistance in the parasite (Perez-Victoria *et al.*, 2003).

Because there are very few drugs in the pipeline, resistance to first-line drug(s) has a very big impact on the treatment of leishmaniasis. Therefore, an understanding of the molecular and biochemical mechanisms of clinical resistance is essential, not only for the development of molecular probes or PCR-based diagnostics to monitor the development and spread of drug resistance in the field, but also for rational drug design for the treatment of drug-resistant *Leishmania*. Prevention and circumvention of resistance towards antimonials has become a World Health Organization priority (<http://www.who.int/tdr/diseases/leish/strategy.htm>).

The mechanism of resistance to antimony in the field is unknown, and most of our understanding stems from work on laboratory mutants, mostly of *Leishmania tarentolae*, in which resistance has been introduced *in vitro* by the selective pressure of heavy metals, principally arsenite (Dey *et al.*, 1994; Légaré *et al.*, 1997), and which are found to be cross resistant to Sb(III) (Haimeur *et al.*, 2000; Brochu *et al.*, 2003). While evaluating resistance mechanisms in the field, it should be kept in mind that *L. tarentolae* is quite different in sensitivity to antimony to species that infect mammals (Table 1). Further, the promastigote cell lines selected for Sb(V) resistance may have been selected for resistance to an *m*-chlorocresol preservative instead of Sb(V) (Ephros *et al.*, 1997), as this stage is not sensitive to pentavalent antimonials. Alternatively, Sb(V) preparations could be partially reduced to Sb(III) due to prolonged storage at

acidic pH or in culture media containing thiols (Ferreira Cdos *et al.*, 2003; Frezard *et al.*, 2001). In recent years, our knowledge regarding the mechanism of action of pentavalent antimonials has been generated very rapidly; hence, the present review focuses on the possible mechanisms which may lead to antimony resistance in *Leishmania* and highlights the links between previous hypotheses and the current developments in field studies.

The primary mechanism of resistance is the decrease in the active drug concentration within the parasite cell. The parasite may lower the drug level by a variety of mechanisms, including decreased uptake, increased efflux, inhibition of drug activation, inactivation of active drug by metabolism or sequestration, and finally by developing a bypass mechanism. The mechanism of antimony resistance has been found to be multifactorial.

Uptake of antimony

To be active against *Leishmania*, antimony has to enter the host cell, cross the phagolysosomal membrane and act against the intracellular parasite.

The route of entry of antimonials [Sb(V)] into *Leishmania* (or into macrophages) is not well understood, although pentavalent arsenate [As(V)], a metal related to Sb(V), is known to enter via a phosphate transporter (Rosen, 2002). The transport of antimony was first studied by using [¹²⁵Sb]Pentostam [Sb(V)] in both stages of the *Leishmania mexicana* and *Leishmania donovani* parasites (Berman *et al.*, 1987b; Croft *et al.*, 1981), but more recently MS approaches have been used to demonstrate the accumulation of two forms of antimony, i.e. Sb(V) and Sb(III), in both stages of the parasite. In a number of species, the accumulation of Sb(V) is higher in axenic amastigotes than in promastigotes (Brochu *et al.*, 2003; Croft *et al.*, 1981). It has been speculated that Sb(V) enters via a protein that recognizes a sugar moiety-like structure shared with gluconate, as gluconate has been shown to inhibit competitively the uptake of Sb(V) in axenic amastigotes (Brochu *et al.*, 2003).

Table 1. Sensitivities to tri- and pentavalent antimonials of promastigote and amastigote stages of *Leishmania* species

ND, Not done. Sb(V) in the form of SAG containing pentavalent antimony; Sb(III) in the form of potassium antimony tartrate containing trivalent antimony.

Species (strain)	Sensitivity to:				Reference
	Pentavalent antimony [Sb(V)] (µg ml ⁻¹)		Trivalent antimony [Sb(III)] (µg ml ⁻¹)		
	Promastigote	Amastigote	Promastigote	Amastigote	
<i>L. donovani</i> (Dd8)	946.52	117.3289	31.46 ± 4.56	ND	Ashutosh <i>et al.</i> (2005)
<i>L. donovani</i> (1SR)	5230 ± 982	19.3 ± 2.3	13 ± 2.4	3.6 ± 0.29	Lira <i>et al.</i> (1999)
<i>L. amazonensis</i>	410 ± 72	270 ± 50	1.58 ± 0.65	32.22 ± 8.35	Sereno & Lemesre (1997)
<i>L. mexicana</i>	470 ± 50	220 ± 30	21.82 ± 2.84	100.24 ± 9.51	Sereno & Lemesre (1997)
<i>L. infantum</i>	248 ± 36	134 ± 45	35.08 ± 5.2	3.8 ± 2.5	Sereno & Lemesre (1997)
<i>L. tarentolae</i>	37 ± 3	ND	0.1 ± 0.03	ND	Haimeur & Ouellette (1998)

Further, neither As(V) nor phosphate can compete with the uptake of Pentostam, ruling out the possibility of the use of an As(V) transporter by Sb(V). Although Sb(V) is accumulated by both stages of the parasite, at pharmacological concentrations, it has no antileishmanial activity (Roberts & Rainey, 1993; Roberts *et al.*, 1995; Sereno & Lemesre, 1997; Sereno *et al.*, 1998). However, in some studies, axenic amastigotes have been found to be as sensitive to Sb(V) as intracellular parasites (Callahan *et al.*, 1997; Ephros *et al.*, 1999; Shaked-Mishan *et al.*, 2001). This discrepancy needs to be resolved. Indeed, several factors, such as species, axenization status, pH and thiol concentration, are likely to affect the drug assay and/or the rate of Sb(V) reduction (Carrio *et al.*, 2000; Ferreira Cdos *et al.*, 2003). The high antileishmanial activity of Sb(III) against both stages of *Leishmania* and the selective activity of Sb(V) against the intracellular parasite further support the hypothesis that the reduction of Sb(V) to Sb(III) is necessary for activity.

Sb(III) also accumulates in *Leishmania* amastigotes and promastigotes. The differential accumulation of Sb(III) and Sb(V) in both stages of the parasite suggests that the two forms of antimony do not enter by the same route. Interestingly, the accumulation of Sb(III) is competitively inhibited by the related metal As(III), whereas the accumulation of Sb(V) is not (Brochu *et al.*, 2003). This strongly suggests that Sb(III) and As(III) enter the cell by the same route as that in yeasts and mammals (reviewed by Rosen, 2002).

In both prokaryotes and eukaryotes (yeast and mammalian), aquaglyceroporins (AQPs) are known to transport trivalent metalloids (Tsukaguchi *et al.*, 1998, 1999; Sanders *et al.*, 1997; Liu *et al.*, 2002; Wysocki *et al.*, 2001). Recently, in *Leishmania*, an AQP (AQP1) has been identified and demonstrated to mediate the uptake of trivalent antimony in the parasite (Gourbal *et al.*, 2004). Transfection of AQP1 is also able to sensitize a Sb(V)-resistant field isolate of *L. donovani* to sodium stibogluconate (SAG), due to increased accumulation of As(III) and Sb(III), thereby indicating its role in natural antimony resistance. Overexpression of AQP1 also renders parasites hypersensitive to Sb(III). Interestingly, this observation has been confirmed by a recent differential gene expression study, in which the expression of AQP1 was down-regulated at both the promastigote and the intracellular amastigote stage in antimony-resistant clinical isolates from Nepal (Decuypere *et al.*, 2005). The mRNA abundance of AQP1 has also been shown to be decreased in antimony-resistant mutants of several *Leishmania* species (Marquis *et al.*, 2005).

The interspecies differences in sensitivity towards Sb(V) and Sb(III) have not been shown to be correlated with differences in the accumulation of antimony (Brochu *et al.*, 2003), which is another very interesting area of research. One possibility for the higher sensitivity of amastigotes towards Sb(V) is a higher accumulation of

Sb(V), which emphasizes the need to study the critical step, i.e. the reduction of Sb(V) within the parasite.

Mechanism of action, and reduction of the metal

Although antimonials have been in clinical use as the first-line treatment for about 60 years, there are still some crucial aspects of Sb(V) metabolism in *Leishmania* that remain uncharacterized. Early work suggested that antimonials probably act by inhibiting glycolysis and fatty acid β -oxidation (Berman *et al.*, 1985, 1987a). However, specific targets in these pathways have not been identified. Recent evidence has shown that antimony kills the parasite by a process of apoptosis involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of membrane (Sereno *et al.*, 2001; Sudhandiran & Shaha, 2003; Lee *et al.*, 2002). However, these effects do not operate via a classical caspase-mediated pathway. Further, Sb(III) has also been shown to inhibit trypanothione reductase (TR) (Cunningham *et al.*, 1994) and glutathione synthetase (Wyllie & Fairlamb, 2006) *in vitro*. Exposure to Sb(III) also results in rapid efflux of trypanothione (TSH) and GSH from isolated amastigotes and promastigotes *in vitro* (Wyllie *et al.*, 2004). Therefore, antimonials may compromise the redox potential of the cell by inducing loss of intracellular thiols coupled with accumulation of disulphides, and by inhibiting TR (Cunningham & Fairlamb, 1995). In animal infections, the mode of action of Sb(V) depends on host factors that also include T-cell subsets and cytokines (reviewed by Murray, 2001). Stibogluconate has also been found to be a potent inhibitor of protein tyrosine phosphatase, which leads to an increase in cytochrome response (Pathak & Yi, 2001).

As Sb(III) is highly active against both stages of the parasite, and Sb(V) is active mostly against intracellular amastigotes (and in some cases against axenic amastigotes also) (Table 1), it is generally agreed that Sb(V) needs to be activated to Sb(III). Further, the site (macrophage or amastigote) and the exact mechanism of reduction (enzymic or non-enzymic) are still unclear. One study has shown that axenically grown amastigotes, but not promastigotes, reduce Sb(V) to Sb(III) (Shaked-Mishan *et al.*, 2001), while other studies have suggested that reduction most likely takes place in the macrophages (Roberts & Rainey, 1993; Sereno *et al.*, 1998). Interestingly, Sb(V) at concentrations up to 1 mg ml⁻¹ has no effect on the growth and viability of macrophages, but Sb(III) even at ~25 µg ml⁻¹ kills 50 % of THP-1 macrophages (Wyllie & Fairlamb, 2006). Since IC₅₀ values for axenic amastigotes vary from 3.2 to 100 µg Sb(III) ml⁻¹ depending upon the *Leishmania* species (Table 1), it is likely that macrophages do not reduce Sb(V) to Sb(III) at a significant level, and that the conversion occurs at both sites, i.e. macrophage and parasite, generating higher lethal concentrations of Sb(III) within the parasite. Certainly a proportion of Sb(V) may be converted to Sb(III) in human (Burguera *et al.*, 1993; Goyard *et al.*, 2003) and animal models (Lugo de Yarbuh *et al.*, 1994).

Both reduced GSH and reduced TSH have been found to be responsible for non-enzymic reduction of Sb(V) to Sb(III) (Ferreira Cdos *et al.*, 2003; Frezard *et al.*, 2001; Yan *et al.*, 2003a, b). The conditions which are found within the phagolysosome in which *Leishmania* resides, i.e. acidic pH (pH 5) and elevated temperature (37 °C), favour this conversion. Further, all antimony is reduced when the molar ratio of GSH:Sb(V) is equal to or more than 5:1, indicating a stoichiometry of 5:1 in the reaction between GSH and Sb(V). As the rate of reduction is very low, the physiological relevance of this conversion is still open to question. Interestingly, promastigotes contain higher intracellular concentrations of TSH and GSH than amastigotes (Ariyanayagam & Fairlamb, 2001; Wyllie *et al.*, 2004), and both stages maintain an intracellular pH value close to neutral (Glaser *et al.*, 1988). The non-enzymic reduction of Sb(V) to Sb(III) fails to account for the insensitivity of promastigotes to Sb(V).

In bacteria and yeast, metal reduction can be mediated by enzymes (Rosen, 2002), and this may be the case in *Leishmania* too. Shaked-Mishan *et al.* (2001) have reported that the reduction of Sb(V) to Sb(III) occurs in both stages of the parasite, but that the activity is much higher in the amastigote stage, which explains the severalfold higher sensitivity of the amastigote to Sb(V) and the role of reduction in sensitization of the parasite. Further, the ability to reduce Sb(V) to Sb(III) is lost in Pentostam-resistant mutants, supporting the role of reducing activity in antimony resistance.

Recently, a parasite-specific enzyme, namely thiol-dependent reductase (TDR)1 has been shown to catalyse the enzymic reduction of pentavalent antimonials to trivalent (Denton *et al.*, 2004). The enzyme is a tetramer protein, containing domains of the omega class of the glutathione S-transferases (GSTs), and uses GSH as the reductant. Although TDR1 has been found to be highly abundant in the amastigote stage of the parasite, a direct relationship between the enzyme activity and antimony sensitivity in *Leishmania* amastigotes cannot be established.

Arsenate reductases are ubiquitous in prokaryotes and eukaryotes, and are essential for conferring resistance to arsenate (Mukhopadhyay & Rosen, 2002). ScAcr2p is an arsenate reductase homologue in *Saccharomyces cerevisiae* that reduces arsenate to arsenite, both *in vivo* and *in vitro* (Mukhopadhyay & Rosen 1998, 2001; Mukhopadhyay *et al.*, 2000). Recently, the arsenate reductase homologue LmACR2 from *Leishmania major* has been identified and characterized. The enzyme has been shown to catalyse the reduction of Sb(V), thus increasing the sensitivity of *Leishmania* cells to Sb(V) (Zhou *et al.*, 2004). Like ScAcr2p, it also requires GSH and glutaredoxin as co-factors for enzyme activity and is inhibited by As(III), Sb(III) and phenylarsine oxide. Purified LmACR2 also has the capacity to reduce both As(V) and Sb(V). However, unlike ScAcr2p, structurally LmACR2 is a monomer. LmACR2 functionally complements the arsenate-sensitive

phenotype of an *arsC* deletion strain of *Escherichia coli* or a ScACR2 deletion strain of *S. cerevisiae*, confirming its role. Most importantly, transfection of LmACR2 in *Leishmania infantum* promastigotes augments Pentostam sensitivity in intracellular amastigotes, confirming its physiological significance. It is also possible that more than one mechanism is responsible for drug activation.

As macrophages do not efficiently reduce Sb(V) to Sb(III) (Wyllie & Fairlamb, 2006), parasites are not exposed to lethal amounts of Sb(III) within macrophages; therefore, reduction of pentavalent antimony becomes the critical event, and the loss of reductive activity in the parasite could lead to resistance. However, this has yet to be shown in clinical resistance.

Efflux of the drug

As a resistance mechanism, the efflux of a drug or its active derivative is very common in bacteria, yeasts and fungi, and various pathogenic protozoa, e.g. *Plasmodium falciparum*, *Entamoeba histolytica*, *Giardia lamblia*, *Trypanosoma cruzi*, and *Trichomonas vaginalis*. This may be the case in *Leishmania* too. There are two types of ABC transporter known to be responsible for multi-drug resistance (MDR) in cancer cells: P-glycoprotein (P-gp) and multi-drug resistance-related protein (MRP). P-gp is encoded by the *mdr1* gene, which confers resistance to many hydrophobic drugs (MDR), and is characterized by reversion with verapamil and cyclosporine A. In *Leishmania*, MRP also confers MDR, although this cannot be reversed by conventional MDR modulators; the protein responsible is known as MRP1.

In *Leishmania*, both classes of ABC transporters have also been reported to be amplified in various species in response to different drugs under laboratory conditions (reviewed by Ouellette *et al.*, 1998, 2001; Leandro & Campino, 2003). The *Leishmania* MDR1 confers resistance to drugs such as daunorubicin and vinblastine, but there is no evidence of a relationship between the MDR1 protein and antimony resistance (reviewed by Ouellette *et al.*, 2001; Klokouzas *et al.*, 2003).

Analysis of the complete *Leishmania* genome (<http://www.genedb.org>) has revealed eight putative protein homologues belonging to the MRP1 family, known to be involved in thiol-associated efflux and metal resistance in mammalian cells (reviewed by Brost & Elferink, 2002; Haimeur *et al.*, 2004). Two of them appear to be involved in antimony resistance in the parasite. The first one is PGPA (renamed as MRPA). However, *Leishmania* MRPA is functionally distinct from mammalian MRP, as resistance is not conferred to pentavalent antimonials, zinc and cadmium, or the typical multi-drug-resistant P-gp substrates vinblastine and puromycin (Ellenberger & Beverley, 1989). The gene has been found to be amplified in a number of laboratory mutants of *Leishmania* species selected for resistance to Sb(III), Sb(V) and As(III) (Callahan & Beverley, 1991; Ferreira-Pinto *et al.*, 1996; Haimeur *et al.*,

2000; Légaré *et al.*, 2001; Ouellette *et al.*, 2001). Its role in antimony resistance has been confirmed by transfection studies (Callahan & Beverley, 1991; Papadopoulou *et al.*, 1994; Légaré *et al.*, 1997). However, this transporter is not responsible for the drug efflux across the plasma membrane. Rather, it confers resistance by sequestration of metal–thiol conjugates, a mode of metal detoxification in yeast cells (Légaré *et al.*, 2001; Rosen, 2002; Tamas & Wysocki, 2001). MRPA is localized in membrane vesicles that are close to the flagellar pocket, the site of endo- and exocytosis in the parasite (Légaré *et al.*, 2001). Overexpression of MRPA has been reported to decrease influx of antimony rather than increase efflux (Callahan *et al.*, 1994), and this may be due to a dominant negative effect through interaction with other membrane proteins. Thus, MRPA is an intracellular transporter rather than an efflux transporter, thereby suggesting that MRPA may play a major role in antimony resistance (Weise *et al.*, 2000; Webster & Russel, 1993).

Recently, it has been shown by DNA microarray that MRPA is overexpressed in the axenic amastigote stage of Sb(III)-resistant *L. infantum* (El Fadili *et al.*, 2005). Transfection of MRPA confers Sb(III) resistance upon promastigotes and Sb(V) resistance upon the intracellular stage of *L. infantum*. However, MRPA has not been found to be upregulated in a comparative transcriptomic study of antimony-resistant *L. donovani* field isolates (Decuypere *et al.*, 2005). Further, no reports are available regarding the amplification of ABC transporter gene(s) in field isolates. Thus, it is still of great interest to determine whether or not drug-resistant field isolates adopt the same strategies to resist antimony as those of laboratory mutants.

Recently, a second ABC transporter protein, PRP1, involved in antimony resistance has been isolated by functional cloning selecting for pentamidine resistance (Coelho *et al.*, 2003). This gene has been shown to confer cross-resistance to antimony. The localization of this protein and the mechanism by which it confers resistance remain to be determined.

Another transporter that confers antimony resistance by an active extrusion system independent of MRPA is also present in *L. tarentolae* laboratory mutants (Dey *et al.*, 1994). Using everted vesicles enriched for the plasma membrane, it has been shown that a metal efflux pump is present in the *Leishmania* plasma membrane. Like MRPA, this efflux pump also recognizes the metal conjugated to thiols such as GSH and TSH (Mukhopadhyay *et al.*, 1996) and requires ATP. The identity of this efflux pump is still unknown, even 10 years after its discovery. Further, it also appears that this efflux system does not play a significant role in antimony resistance, as the transport kinetics of the vesicles prepared from sensitive and resistant isolates are similar (Dey *et al.*, 1996).

Thiol metabolism

Thiol metabolism has a central role in the maintenance of an intracellular reducing environment so that the cell can

defend itself against the damage caused by oxidative stress inside the macrophage, oxidants, certain heavy metals and, possibly, xenobiotics (Meister & Anderson, 1983). As antimony causes oxidative stress (Lecureur *et al.*, 2002), a reducing environment within the cell and the presence of thiols become important for antimony resistance.

Arsenite- or antimony-resistant laboratory mutants of all *Leishmania* species exhibit significantly increased levels of intracellular thiols, namely cysteine, GSH, spermidine and TSH, suggesting a role for thiols in resistance (Mukhopadhyay *et al.*, 1996; Legare *et al.*, 1997; Haimeur *et al.*, 2000). TSH, the major thiol, is found only in trypanosomatids, and is a conjugate of GSH and spermidine (Fairlamb & Cerami, 1992). The synthesis of these two precursors determines the level of TSH. The γ -GCS gene, encoding γ -glutamylcysteine synthetase, which catalyses the rate-limiting step in GSH biosynthesis, has been found to be amplified in arsenite-resistant *L. tarentolae* (Grondin *et al.*, 1997), while the gene *ODC*, which encodes ornithine decarboxylase, an enzyme involved in the regulation of spermidine biosynthesis, is also overexpressed (Haimeur *et al.*, 1999; Guimond *et al.*, 2003). This suggests that a lowering of intracellular thiol concentrations may result in the attenuation of the resistant phenotype. This proposed hypothesis is confirmed by inhibition studies. The inhibition of the γ -GCS and *ODC* genes by their specific inhibitors, L-buthionine-(SR)-sulphoximine (BSO) and DL- α -difluoromethylornithine (DFMO), respectively, results in the reversal of arsenite or antimony resistance in laboratory mutants (Arana *et al.*, 1998; Grondin *et al.*, 1997; Haimeur *et al.*, 1999; Légaré *et al.*, 2001). Although the combination of BSO and DFMO sensitizes the resistant cells, the residual level of resistance is still higher than that in wild-type cells, suggesting that GSH and TSH alone are not sufficient to confer metal resistance. Overexpression of either *ODC* or γ -GCS in *L. tarentolae* wild-type cells results in increased thiol levels, almost equivalent to those of resistant mutants, but the transfectants do not exhibit arsenite resistance (Grondin *et al.*, 1997). While co-transfection of *ODC* or γ -GCS with MRPA in wild-type cells results in arsenite resistance (Haimeur *et al.*, 1999, 2000), this acquired resistance in transfectants is also reversed by the thiol depletor BSO (El Fadili *et al.*, 2005). This therefore establishes that MRPA and increased TSH concentrations act synergistically, and that TSH availability is the limiting factor in both the transport of drug conjugates and resistance to arsenite and/or antimony (Ouellette *et al.*, 1991). In *Leishmania tropica* and *Leishmania mexicana* cell lines, an increase in TSH is not associated with either the amplification of γ -GCS or overexpression of *ODC* (Légaré *et al.*, 1997).

Interestingly, resistance to Sb(V) in *L. donovani* clinical isolates (India) is also reversed in animal models by treatment with BSO (Carter *et al.*, 2003, 2005). It is also noteworthy that in these resistant isolates, the expression of γ -GCS is also increased significantly. However, in another study on *L. donovani* isolates from Nepal,

expression of γ -GCS and ODC was significantly decreased in resistant isolates (Decuypere *et al.*, 2005). Therefore, there is a need to study the level of thiols in clinical isolates and determine their role in natural antimony resistance.

Recent studies have shown that antimony-resistant isolates down-regulate the expression of γ -GCS of macrophages (Carter *et al.*, 2006), probably by down-regulating host NF- κ B, which is known to regulate γ -GCS expression (Jang & Surh, 2004). This would result in the reduction of intramacrophage GSH levels and promote an intracellular oxidative environment (Wyllie & Fairlamb, 2006), thereby minimizing the intramacrophage reduction of Sb(V) to its toxic form Sb(III). These results clearly indicate that SAG resistance in *L. donovani* is associated with manipulation of both host and parasite thiol levels.

Spontaneous formation of Sb(III) complexed with GSH or TSH, or both, has already been demonstrated by proton nuclear magnetic resonance spectroscopy (Sun *et al.*, 2000; Yan *et al.*, 2003) and by MS (Mukhopadhyay *et al.*, 1996). Since GST is elevated in mammalian cells selected for resistance to arsenite (Lo *et al.*, 1992), it has been proposed that GST mediates the formation of metalloid thiol pump substrates in *Leishmania* species also. However, in *Leishmania*, GST is not detectable; rather, a related trypanothione S-transferase activity is observed (Vicker & Fairlamb, 2004).

Thus, the thiols have a dual role in antimony resistance, i.e. sensitization of the parasite by the reduction of pentavalent to trivalent antimony, and promoting resistance by forming conjugates with trivalent antimony for efflux and/or sequestration.

Changes in the cytoskeleton

Microtubules are dynamic cytoskeleton polymers consisting of repeating α -/ β -tubulin heterodimers along with γ -tubulin, and are vital for cell shape, growth and differentiation of *Leishmania* (Chan *et al.*, 1991). Since arsenite has high affinity to bind tubulins and can inhibit tubulin polymerization (Ramirez *et al.*, 1997; Jayanarayan & Dey, 2004), acquisition of resistance to arsenite or antimony may cause changes in cytoskeleton proteins.

Recent studies have shown that the expression of α -tubulin is similar in both wild-type promastigotes and arsenite-resistant mutants. Differentiation into axenic amastigotes causes down-regulation in resistant mutants compared with the wild-type (Prasad *et al.*, 2000). A twofold increased sensitivity of a mutant resistant to Paclitaxel (known to promote tubulin assembly) is found to decrease the expression of α -tubulin in arsenite-resistant mutant promastigotes (Prasad *et al.*, 2000). On the other hand, the expression level of β -tubulin is higher in both stages of an arsenite-resistant mutant than in the wild-type (Jayanarayan & Dey, 2002), while γ -tubulin expression is upregulated in the amastigote stage only, and is unaltered in the

promastigote stage. Although Paclitaxel treatment significantly increases the expression of β -tubulin in resistant promastigotes, it has no effect on γ -tubulin expression in either strain, either before or after differentiation (Jayanarayan & Dey, 2002). Further, arsenite treatment has been shown to decrease the expression of α - and β -tubulin in wild-type promastigotes, while expression remains unaltered in an arsenite-resistant mutant (Jayanarayan & Dey, 2004). Since tubulin synthesis is regulated by the unpolymerized tubulins, and arsenite has been shown to inhibit microtubule polymerization in the parasite, arsenite may decrease the synthesis of tubulins by inhibiting polymerization. It is noteworthy that phosphorylation of α - and β -tubulin is highly increased in the arsenite-resistant mutant (Prasad & Dey, 2000). Phosphorylation of tubulins could directly affect the dynamics of tubulin assembly and could regulate and affect several signal-transduction pathways (Gundersen & Cook, 1999); therefore, tubulins have an important role in metal resistance.

Other mechanisms

The levels of heat-shock proteins of varying molecular masses are known to increase in the presence of arsenite (Lawrence & Robert-Gero, 1985). Overexpression of Hsp70 is capable of bestowing a heat-inducible increase in resistance to peroxide (Miller *et al.*, 2000). Recently, Hsp70 and Hsc70 have been identified by functional cloning (Brochu *et al.*, 2004). However, transfection of an HSP gene does not confer resistance directly, but rather increases the tolerance of the cell to metals. Further, it is still not known how Hsp70 provides resistance and whether this mechanism operates in field isolates also.

It is known that antimony kills *Leishmania* by inhibiting enzymes of the glycolytic pathways, and by effects on thiol redox potential, DNA fragmentation and programmed cell death (Berman *et al.*, 1985, 1987a; Sereno *et al.*, 2001; Wyllie *et al.*, 2004). It seems that the modes of action of antimony and NO are closely correlated, because both act by oxidative stress, depletion of enzymes of energy pathways, and eventual apoptosis (Holzmuller *et al.*, 2002). Like antimony, NO also induces the expression of stress proteins (Hsp70, Hsp81 and Hsp65), and this is thought to be a protective mechanism against its toxic effect (Adhuna *et al.*, 2000). Interestingly, Sb(III)-resistant *L. infantum* amastigotes have been found to be cross resistant to NO when NO is supplied extracellularly by an NO donor or intracellularly via macrophage activation (Holzmuller *et al.*, 2005).

In another study, *L. tarentolae* promastigotes selected for resistance to SAG, and cross resistant to other antimony-containing drugs and arsenite, showed amplification of a locus containing an ORF of 770 aa. However, the gene for this ORF does not have significant homology with any database sequence (Haimeur & Ouellette, 1998); therefore, it is still uncharacterized.

Conclusions

Drug resistance is a major impediment to successful treatment of leishmaniasis (Guerin *et al.*, 2002; Sundar *et al.*, 2000). Unfortunately, the analysis of resistance mechanisms in field isolates has lagged behind studies of laboratory-derived resistant mutants. Resistance is an interplay between the uptake, efflux and sequestration of active molecules (Croft *et al.*, 2006). In laboratory-derived resistant cell lines, *Leishmania* cells are exposed to Sb(III),

the active molecule; therefore, the resistance mechanisms involved are mainly based on decreasing the concentration of the drug within the cell. This could be achieved either by decreasing Sb(III) uptake by decreased expression of AQP1 or by increasing efflux/sequestration of the active drug in conjugation with thiols by increasing the levels of thiols and by amplification of transporters. Indeed, both mechanisms have been demonstrated in several resistant laboratory mutants (El Fadilli *et al.*, 2005; Grondin *et al.*, 1997; Haimeur *et al.*, 2000; Lgar *et al.*, 1997; Marquis *et al.*, 2005)

Fig. 1. Schematic representation of proposed mechanism of antimony resistance in *Leishmania*. (a) Laboratory-generated Sb(III)-resistant mutants; (b) natural Sb(V)-resistant clinical isolates. The entry of Sb(V) into the parasite occurs by an unknown transporter, and entry of Sb(III) through AQP1. In Sb(III)-resistant cell lines, the level of TSH is increased due to a severalfold increase in the activity of ornithine decarboxylase (ODC) and γ -glutamylcysteine synthetase (γ -GCS), the rate-limiting enzymes for thiol biosynthesis. The detoxification pathway includes complex formation of Sb(III) with TSH and subsequent sequestration via amplified MRPA and/or efflux by unknown pumps. In natural Sb(V) resistance, a lower expression of ODC putatively leads to lower thiol biosynthesis, thus inhibiting activation of Sb(V). Decreased expression of AQP1 also restricts the entry of Sb(III) into the parasite. Intracellular Sb(III) may be dealt with either by sequestration or by efflux of thiol-Sb(III) conjugates, while that of macrophages is conjugated with GSH and effluxed by host ABC transporters. Other abbreviations: TSH, reduced trypanothione; TS₂, oxidized trypanothione; Sb(TS)₂, conjugate of Sb(III) with trypanothione; MRPA, ABC transporter.

(Fig. 1a). In natural antimony resistance, parasites are exposed to Sb(V), and there is an extra resistance step involved, which is to minimize the conversion of the Sb(V) prodrug to Sb(III), at both the host and the parasite level (Fig. 1b). Based on a few studies conducted on field isolates, it has been demonstrated that the resistant parasite modulates the expression of γ -GCS in the host macrophage (Carter *et al.*, 2006), thereby decreasing the host GSH concentration and also host-mediated conversion of Sb(V) to Sb(III). The parasite also down-regulates the expression of key enzymes for the synthesis of the GSH and spermidine moieties of TSH (Decuypere *et al.*, 2005), thereby decreasing the parasite thiol-mediated activation of the drug. Further, it also restricts the entry of macrophage-generated Sb(III) by lowering the expression of AQP1 (Gourbal *et al.*, 2004), an uptake channel (reviewed by Ouellette *et al.*, 2004). Therefore, it may be proposed that natural resistance to antimony results from a lower accumulation of the active drug within the parasite. Finally, the Sb(III) that enters the parasite may be taken care of either by sequestration or by efflux of thiol-Sb(III) conjugate. However, these steps still need to be confirmed with more isolates. Thus, the mechanism of natural antimony resistance is multifactorial, and may differ from laboratory resistance.

Sb(V)-resistant strains are emerging throughout the Indian subcontinent, and a new generation of molecular-based drug-susceptibility tests is urgently required to set up feasible and efficient monitoring strategies. So far, the only reliable method for monitoring the resistance of isolates is the technically demanding *in vitro* amastigote macrophage model (Carter *et al.*, 2001; Lira *et al.*, 1999), which involves counting the stained parasite. Field isolates expressing reporter genes such as firefly luciferase can greatly facilitate the analysis of resistance (Ashutosh *et al.*, 2005). Further, no molecular markers of resistance are available for currently used antileishmanial drugs, i.e. SAG. These aspects urgently need to be developed to monitor regular drug use, drug response and the spread of resistance.

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